Please cite this article in press as: Badenhorst and Bornscheuer, Droplet microfluidics: From simple activity screening to sophisticated kinetics, Chem (2021), https://doi.org/10.1016/j.chempr.2021.03.003

Chem

CellPress

Preview

Droplet microfluidics: From simple activity screening to sophisticated kinetics

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In this issue of *Chem*, Hess et al. describe a droplet-based microfluidic platform for the acquisition of transient kinetic data of enzyme-catalyzed reactions. Compared with conventional equipment, their platform reduces assay volumes by up to six orders of magnitude and allows up to 9,000 reactions to be screened per minute.

Despite the vast number of enzymes in nature, relatively few have been employed industrially because they usually need to be engineered to meet process requirements.[1] Unfortunately, engineering proteins is difficult because we lack detailed mechanistic understanding of how they work. As Nobel Laureate Frances Arnold said, "When I started engineering proteins, I didn't know how hard it would be."[2] She therefore pioneered the use of directed-evolution strategies that allow us to "engineer" proteins by screening large libraries without actually understanding how they work. Over the past decade, droplet-based microfluidics has become an increasingly popular tool in the development of library screening assays because of its high throughput and low reagent consumption, which allow millions of variants to be screened.[3] However, high-throughput screening methods usually employ end-point assays, and therefore finding the best starting point for subsequent rounds of directed evolution requires that the "hits" be kinetically characterized. In most laboratories, this is a demanding task if more than a handful of hits are identified. So, why not employ the same high-throughput methodology to enzyme characterization? Surprisingly, despite their clear advantages for high-speed kinetic analyses, microfluidic systems are not yet commonly employed for studying the kinetics or thermodynamics of enzyme-catalyzed reactions.[4]

In this issue of *Chem*, Hess et al.[5] now demonstrate that droplet microfluidics can be used for studying the transient kinetics and scanning the thermodynamic profiles of various enzymes (β-galactosidase, horseradish peroxidase, micro-peroxidase, and haloalkane dehalogenase). The authors used temperature-dependent single- and multiple-turnover measurements to determine kinetic and thermodynamic parameters for the individual steps of a complex dehalogenation reaction. Extracting such quantitative thermodynamic parameters would be very hard with conventional techniques, but this is facilitated by the rapid heat transfer between microfluidic droplets and their surroundings.

The significance of their platform can be better appreciated by comparison to traditional high-speed kinetic methods. Despite the convenience and flexibility of the commonly employed microtiter plates, the first few seconds of a reaction are invariably lost as a result of slow sample handling and mixing. Millisecond burst phases, which are common for enzyme-catalyzed reactions, can therefore not be recorded with these tools. Obtaining transient kinetic data requires that a measurement be started almost immediately after very rapid mixing of reagents. About a century ago, this was achieved by the rapid pumping of two solutions through a mixing chamber. At a constant flow rate, the distance between the mixing and observation sites corresponds to the reaction time.[6] Unfortunately, this method consumed vast volumes of reagents stored in 20 L stoneware bottles. Although this was feasible for studies of hemoglobin from blood, the scarcity of enzyme preparations in the 1930s motivated the miniaturization of assay volumes. Simply shrinking channel dimensions and increasing flow rates could not solve the problem because of increasing backpressure. Furthermore, rapid mixing of fluids is complicated by laminar (as opposed to turbulent) flow in microfluidic channels. More importantly, fluid in the center of a channel moves significantly faster than fluid in contact with the channel walls, so an initially sharp band of a reaction mixture is parabolically distorted as it flows through a narrow channel (Figure 1). This process, known as Taylor dispersion, establishes concentration gradients and abolishes the concrete time-distance-concentration relationship that is essential for high-speed kinetic measurements. A major advance was the development of stopped-flow techniques.[7] After two liquids are mixed, flow is stopped abruptly, and the change in absorbance or fluorescence is immediately recorded. This allows data to be collected within 1 ms of mixing. Stopped-flow equipment became the gold standard and has since stood the test of time despite

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https://doi.org/10.1016/j.chempr.2021.03.003

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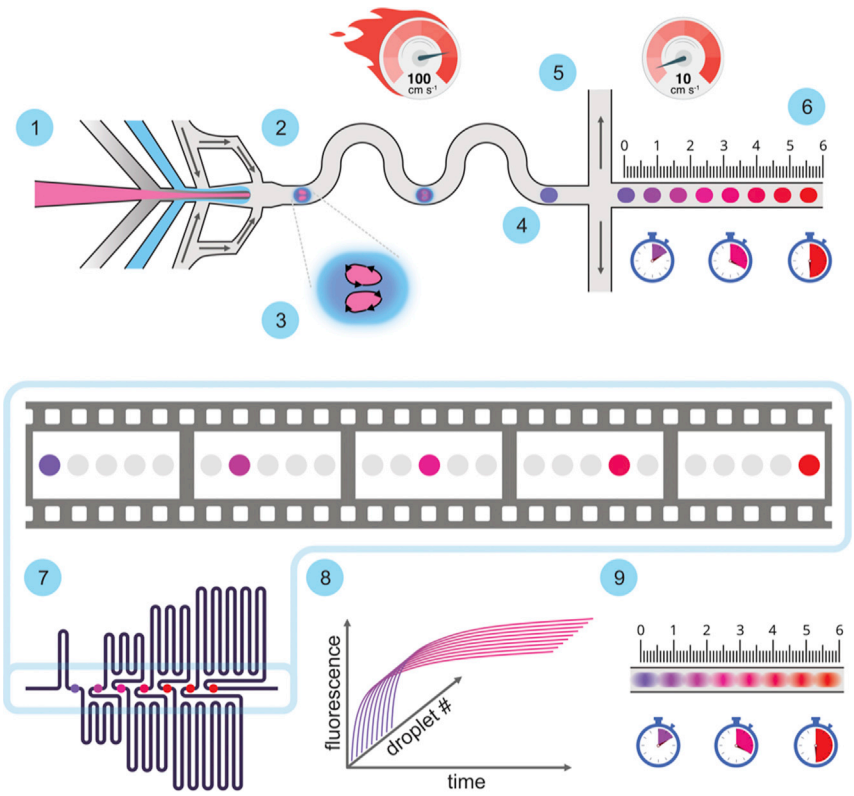

Figure 1. Droplet-based ultrahigh-throughput characterization of transient enzyme kinetics

(1) Substrate and enzyme solutions are injected into the chip with two separate syringes. A buffer stream physically separates the substrate and enzyme from each other prior to droplet formation, preventing premature initiation of the reaction. Changing the flow rates through the syringes, and compensating with a corresponding change in buffer flowrate, can manipulate the concentrations of enzyme and substrate.

(2) Immediately after formation, injection of more spacing oil accelerates droplets to about 100 cm/s through a serpentine channel.

(3) This results in rapid mixing by chaotic advection.

(4) Droplet contents are completely mixed in less than a millisecond such that 90% mixing is achieved in only 800 μs. The authors cleverly demonstrated this rapid mixing by simultaneously injecting a calcium solution and a solution of Fluo-4, which becomes fluorescent upon binding to calcium.

(5) After mixing, the spacing oil between droplets is drained, decreasing droplet velocity to about 10 cm/s. This allows high-quality stroboscopic epifluorescence imaging at about 2,000 frames per second.

(6 and 7) The precise relationship between time and droplet position (accuracy of ±5 μm) (6) allows individual droplets to be tracked by analysis of a series of photographs taken by the highspeed camera (7). The length of the detection channel is increased by a series of switchbacks, allowing the droplets to be imaged for an extended period.

(8) Plotting fluorescence intensity at each of these time points results in time traces for individual droplets, each of which represents an experiment.

(9) Droplet formation is critical to preventing dispersion, which would form concentration gradients and distort the essential relationship between distance and time and thus make it impossible to track individual reaction conditions over significant distances.

two major limitations. First, modern micro-volume stopped-flow equipment can generate data by using as little as 10 μL of stock solution per experiment, but this volume is still large considering the numerous reactions and large datasets needed for detailed kinetic characterization. Second, the method is intrinsically low in throughput because of its discontinuous mix-stop-measure-repeat nature. Fortunately, the most attractive features of continuous-flow and stopped-flow methods are combined in droplet microfluidic systems, which segment continuously flowing aqueous streams into discrete droplets and thus prevent Taylor dispersion. Therefore, the droplets can be analyzed at different locations, which correspond to different reaction time points.

The signal for a large number of droplets (for each condition) can be averaged, resulting in high sensitivity, low detection limits, and more reliable data. However, Hess et al.'s system goes far beyond mere accumulation of many replicate data points for each reaction condition and time. A key innovation is the use of a cylindrical lens to focus excitation light on a narrow strip along the length of a straight observation channel, unlike the single submicron spot typically employed by high-throughput droplet-sorting platforms. Thus, the use of epifluorescence imaging enables individual droplets to be tracked as they move through a long observation channel (Figure 1). This means that an entire fluorescence-versus-time dataset (i.e., an experiment) is generated with less than 5 pL of reagents, corresponding to over 200 million experiments per milliliter of reagents. For the same number of reactions, conventional micro-volume stopped-flow equipment would require over 100 of Hartridge and Roughton's antique 20 L stoneware bottles full of reagents. This is a truly remarkable achievement. In fact, even at its lowest performance, Hess et al.'s system completes an experiment in 1 min while consuming less than 1 μL of sample. This makes it fair to say that at its worst, their system is at least an order of magnitude better than the state of the art.

The authors demonstrated that integration of their platform with global data modeling and molecular dynamics simulations can provide insights into the mechanistic differences between

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variants of an enzyme. For example, engineering tunnels of an enzyme has long been known to influence catalysis by controlling the transport of substrate and product molecules between the active site and the bulk solvent. However, using their high-speed kinetic data, the authors now show that tunnel mutations also influence the rates of the chemical steps and can result in a switch in the rate-limiting step of the reaction. Their results also suggest that even for the same variant, the reaction mechanism can depend on the substrate. For instance, whereas the reaction with 1,2-dibromoethane is driven by a fast chemical step, the dehalogenation of the bulky fluorogenic substrate 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-COU) is driven by the formation of a strong enzyme-substrate complex, which results from the entropically favored desolvation of the enzyme active site. There is a common understanding in protein engineering that "you get what you screen for," cautioning against the use of fluorogenic and chromogenic surrogate substrates given that these can lead to the identification of variants that produce color but not the actual target compound. We believe that this result from Hess et al. not only clearly demonstrates the bias of screening with fluorogenic substrates but also reminds us that the same concept applies in the subsequent study of the high-speed kinetics of enzyme variants.

So, what does this all mean for protein engineers? Modern enzyme engineering strives to create "small but smart"[8] libraries—designed on the basis of information-intensive bioinformatic and computational tools such as 3DM[8,9] or FRESCO[10]—dramatically decreasing the number of variants that have to be screened. We are confident that over time, the integration of microfluidic high-throughput characterization of enzyme kinetics with computational methods will further reform our understanding of protein function and drive protein engineering ever further from directed evolution toward actual rational engineering. We are very excited about a future where high-throughput screening will be transformed into high-throughput characterization given that there is no reason to believe the platform reported by Hess et al. cannot eventually be adapted to the high-throughput kinetic characterization of entire mutant libraries. High-throughput experimentation is here to stay, even in the age of computational biology.

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